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INFLUENCE OF CYCLOHEXIMIDE AND 1,25-DIHYDROXY-VITAMIN D3 ON MIT--ETC(U)
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$1,25(OH)_2D_3$ increases cell permeability to calcium. This increase is not mediated by proteins sensitive to cycloheximide or actinomycin D inhibition. It is proposed that CaBP may associate with intracellular membranes and organelles to prevent intracellular calcium accumulation and the potential cytotoxic effects of such accumulation. In support of this hypothesis, the amount of mitochondrial mineralization in chick intestinal cells was markedly increased by $1,25(OH)_2D_3$ treatment when CaBP synthesis was simultaneously blocked by cycloheximide treatment. Mineral in membrane vesicles was increased by

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1,25(OH)$_2$D$_3$ treatment when CaBP synthesis was simultaneously blocked by cycloheximide treatment. Mineral in membrane vesicles was increased by 1,25(OH)$_2$D$_3$ treatment, but was blocked by simultaneous treatment with cycloheximide.

OH2 D3

Influence of Cycloheximide and 1,25-Dihydroxyvitamin D_3 on Mitochondrial and Vesicle Mineralization in the Intestine

ROBERT L. MORRISSEY,[1] DAVID T. ZOLOCK,[2]
PAUL W. MELLICK, AND DANIEL D. BIKLE

Letterman Army Institute of Research, Presidio of San Francisco 94129

RUNNING TITLE: Cycloheximide and 1,25$(OH)_2D_3$

TABLE OF CONTENTS CATEGORY: PATHOLOGICAL PHYSIOLOGY

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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SEND CORRESPONDENCE TO: MAJ Robert L. Morrissey

Pathology Division

U.S. Army Medical Research Institute of

Infectious Diseases

Fort Detrick, MD 21701

The metabolic pathway of vitamin D has been extensively studied in recent years. Vitamin D is hydroxylated in the 25 position (1) by the liver (2-4) and in the 1 position by the kidney (5). The mechanism of action of the resulting 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] on the target tissues remains to be defined fully, but several pertinent observations have been made: (a) a cytosol-binding protein for $1,25(OH)_2D_3$ has been reported (6-8); (b) highly specific binding sites for $1,25(OH)_2D_3$ occur in the nucleus (9); (c) a specific mRNA for calcium binding protein (CaBP) is formed in rachitic chick intestine after treatment with $1,25(OH)_2D_3$ (10); (d) polyribosomes from $1,25(OH)_2D_3$-treated rachitic chicks synthesize CaBP (11); and (e) CaBP is synthesized in columnar epithelial cells of intestine of rachitic chicks after treatment with $1,25(OH)_2D_3$ (12-14). These observations have led various authors (15, 16) to propose models which hypothesize that these events lead to and account for the change in intestinal calcium transport rate which also occurs after treatment with $1,25(OH)_2D_3$. However, the following observations are not readily explained by the current models: (a) the time of first appearance of CaBP in intestine does not precede increased calcium transport rate after $1,25(OH)_2D_3$ treatment (12, 13); (b) the enhanced rate of intestinal calcium transport after $1,25(OH)_2D_3$ treatment dissipates much faster than the induced CaBP (12, 13); and (c) CaBP synthesis is decreased or completely blocked when rachitic chicks receive actinomycin D or cycloheximide with $1,25(OH)_2D_3$, but such treatment does not prevent the increase in calcium transport across the

intestine (17). Therefore, we propose a mechanism of action for $1,25(OH)_2D_3$ on intestine wherein the calcium transport changes are independent of new protein synthesis, or at least any protein synthesis which is blocked by cycloheximide or actinomycin D.

Although CaBP does not appear to be essential for the intestinal cell to transport calcium, it does appear to be required to maintain a low intracellular calcium concentration. This conclusion was reached from the following observations: (a) treatment of rachitic chicks with $1,25(OH)_2D_3$ resulted in increased intracellular accumulation of calcium initially and prior to the appearance of CaBP (13); (b) at the onset of CaBP synthesis, intracellular calcium accumulation began to decrease (18); and (c) if CaBP synthesis was blocked by cycloheximide treatment, this decrease did not occur (18). These observations were made via <u>in vivo</u> radiotracer studies which did not address the question of which intestinal cells were accumulating calcium and where the calcium was accumulating in those cells. The current study was designed to: (a) test the hypothesis that the intestinal mitochondria are a major site of intracellular calcium accumulation, the extent of which correlates negatively with the presence of CaBP, and (b) evaluate the influence of $1,25(OH)_2D_3$ and cycloheximide on mineral content of other intracellular organelles. Our approach was to examine mitochondrial and vesicle mineralization at various sites along the villus at 18 hr after $1,25(OH)_2D_3$ treatment in the presence or absence of concomitant treatment with cycloheximide at a dosage schedule known to block CaBP synthesis. CaBP was present in

absorptive cells along the entire villus 18 hr after $1,25(OH)_2D_3$ treatment (14).

Methods. Male, Leghorn chicks were fed a vitamin D-free diet containing 0.43% calcium and 0.3% phosphorus from the time they were 1-day old until the 17th day. The $1,25(OH)_2D_3$-treated chicks received an oral dose of 62.5 pmoles of $1,25(OH)_2D_3$ in 0.1 ml of dose carrier and the control chicks received only the carrier (0.2% ethanol in propylene glycol). Cycloheximide-treated chicks received an intraperitoneal injection of 50 μg of cycloheximide in 0.1 ml propylene glycol 1 hr before and 3, 7, 11 and 17 hr after the $1,25(OH)_2D_3$ or carrier dose. After the appropriate treatment, chicks were anesthetized with sodium pentabarbitol, and a specimen of intestine was removed and immediately fixed in osmium-pyroantimoniate (3.75% $K_2H_2Sb_2O_7 \cdot 4H_2O$-1.0% OsO_4, pH adjusted to 7.2 with acetic acid), a modification of the fixation procedure reported by Solomon et al. (19). Specimens were fixed for 2 hr, washed 2 times for 30 min each in 10.0% sucrose, held overnight in 10% sucrose, dehydrated by a graded series of ethanols and propylene oxide, and embedded in epon-araldite. Thick sections (1 μ) were stained with methylene blue-azure II and basic fuchsin to identify the most optimally oriented villus for examination. The block was then trimmed for ultramicrotomy. Thin (gold to silver) sections were cut on an LKB III ultramicrotome, collected on formvar-coated slot grids, and photographed through a Hitachi model HS-8F-2 electron microscope at an accelerating voltage of 50 kv. Overlapping low magnification (1000 X) photomicrographs were used to construct a composite picture

of the villus in order to measure the distance from the muscularis mucosa to the site of mitochondrial examination. Photomicrographs taken at 4,600 X magnification were enlarged and printed at a final magnification of 26,154 X to count the number of mineralized granules within mitochondria and the number of mitochondrial cross-sections (approximately 100/site) at the site. The average number of granules/mitochondrion was derived by pooling the data for sites along the villus distal to the crypt region. One villus was evaluated from three chicks in each treatment group.

The number of intracellular membrane vesicles which contained fine granular precipitate was evaluated at each of the sites for mitochondrial mineralization (Fig. 2C). Vesicles which lacked the precipitate were not included in the count. No attempt was made to evaluate the quantity of mineral in a given vesicle. The average number of mineral-containing vesicles was determined for each specimen.

The significance of treatment effects was evaluated by using the Student t test.

Results. The mitochondria in the cells located in the crypt region were heavily mineralized in all four treatment groups. Thus, it was necessary to eliminate those sites from the analysis to avoid masking of the treatment effects on sites along the villus.

The mitochondrial response is illustrated in Fig. 1. The number of mitochondrial cross-sections observed along various villi ranged from 550 to 3598. The number of electron-dense granules per mitochondrial cross-section did not differ significantly

($P > 0.05$) in villi from untreated chicks, and those treated with $1,25(OH)_2D_3$ or treated with cycloheximide alone. However, mitochondrial mineralization was markedly increased ($P < 0.05$) in chicks treated with combined $1,25(OH)_2D_3$ and cycloheximide when compared to either treatment alone. A representative site from each of the four treatment groups is shown in Fig. 2.

The influence of the treatments on vesicle mineralization is illustrated in Fig. 3. The number of mineral-containing vesicles per site did not differ significantly in villi from untreated chicks, chicks treated with cycloheximide only and chicks treated with cycloheximide and $1,25(OH)_2D_3$ ($P > 0.05$). In contrast, there was a marked increase in mineralized vesicles in the villi from chicks treated with $1,25(OH)_2D_3$ alone when compared to the other groups ($P < 0.025$).

Discussion. Morphologic techniques such as electron probe analysis (20), electron microscopy coupled with microincineration (21), autoradiography (21) and pyroantimony precipitation techniques (22) have been used to demonstrate increased movement of calcium into intestinal epithelial cells after vitamin D treatment. In vitro radiotracer techniques (23) have further revealed increased accumulation of calcium after treatment with vitamin D. However, the response is time-dependent when $1,25(OH)_2D_3$ is used to stimulate calcium absorption and calcium accumulation is evaluated by in vivo radiotracer techniques (13, 18). The hypothesis of the present study could not be tested as effectively at a time when intracellular accumulation was increased by $1,25(OH)_2D_3$. With this in mind, we

selected the 18 hr time in this study. Thus, we do not interpret our results to be in conflict with the earlier studies which demonstrate increased calcium accumulation after vitamin D treatment.

The marked elevation of mineral in membrane vesicles 18 hr after treatment with $1,25(OH)_2D_3$ (Fig. 3) infers that the drug increased calcium flux into vesicles. However, this action of $1,25(OH)_2D_3$ was inhibited by cycloheximide (Fig. 3) and thus was apparently not a direct effect, but rather one that was mediated via synthesis of CaBP or some other as yet undefined $1,25(OH)_2D_3$-induced protein. The increased number of mineral-containing vesicles at a time after $1,25(OH)_2D_3$ treatment when total intracellular calcium has returned to normal (18) indicates that the size of the vesicular calcium pool must be quite small relative to the quantity of calcium that mitochondria can accumulate. A potential difference in calcium pool size at the two sites is further suggested by the nature of the precipitate in the respective sites. The precipitate in mitochondria occurred as discrete, relatively large granules consistent with in vivo precipitation while that in vesicles occurred as a more diffuse, fine precipitate consistent with precipitation of soluble or protein bound calcium by the pyroantimoniate during tissue fixation. The effect of $1,25(OH)_2D_3$ with and without cycloheximide treatment on mitochondrial mineralization was the inverse of the effect on mineral content of vesicles. Combined treatment with $1,25(OH)_2D_3$ and cycloheximide resulted in considerably more mitochondrial mineralization (Fig. 3) compared to either treatment alone. Thus, the effect of $1,25(OH)_2D_3$ on mitochondrial mineralization was either direct, a consequence of increased permeability of the

plasma membrane to calcium, or a combination of the two possibilities.

A model is presented as Fig. 4 to represent the actions of $1,25(OH)_2D_3$ on the intestinal epithelial cell. The major concepts added to previous models include the following: (a) An effect of $1,25(OH)_2D_3$ on the mRNA for alkaline phosphatase is supported by the observation that actinomycin D additively enhanced the alkaline phosphatase response to $1,25(OH)_2D_3$ rather than inhibiting it (17). (b) An effect of $1,25(OH)_2D_3$ on the membrane that is independent of protein synthesis is supported by the observation that cycloheximide did not block calcium transport at treatment dosages which completely blocked CaBP synthesis (18). (c) A role of CaBP in prevention of intracellular calcium accumulation by either preventing its mitochondrial accumulation or enhancing its removal from mitochondria is supported by the observations reported herein. Also, in vivo radiotracer studies have shown that $1,25(OH)_2D_3$ increased the intracellular accumulation of calcium initially, but at the onset of CaBP synthesis, calcium accumulation began to decrease. If CaBP synthesis was blocked, the expected decrease in intracellular calcium accumulation did not occur (18). Such a role is also supported by in vitro studies which showed that CaBP caused release of calcium from mitochondria and diminished its uptake by mitochondria (24), and (d) A possible role for CaBP in the enhancement of membrane vesicle calcium uptake is supported by the observation that cycloheximide blocked the increased vesicle calcium content that occurred after $1,25(OH)_2D_3$ treatment.

Although this model takes into account several recent observations, it does not fully explain how $1,25(OH)_2D_3$ alters the calcium transport rate of intestinal absorptive cells. However, we propose the following sequence of events: (a) the initial intestinal response to $1,25(OH)_2D_3$ may be an increase in cell permeability to calcium by an unknown mechanism that is independent of de novo protein synthesis; (b) this increased permeability may result in increased cytosol concentration and thus increased availability of ionic calcium to a pump in the basal border of the cell; (c) in the absence of CaBP the $1,25(OH)_2D_3$-mediated increase in cytosol calcium concentration may result in its accumulation in mitochondria to protect intracytoplasmic organelles from its toxic effects. When CaBP is present it may prevent calcium from associating with mitochondria or enhance its dissociation from them by either a direct effect on the mitochondria or by lowering the cytosol concentration via enhanced uptake by membrane vesicles which later excrete their product at the basal-lateral cell border. CaBP may also have some specific effect on the basal and lateral cell borders to enhance the removal of calcium from the cell and spare the requirement for ATP expenditure and sodium entry into the cell. This possibility is supported to some extent by the observation that CaBP is secreted into the blood from intestine (25). Secretion of CaBP into blood during calcium absorption suggests that CaBP may be present inside the vesicles. However, the nonessentiality of CaBP for increased calcium transport rate argues against this mechanism being responsible for the initial increase in cell permeability to calcium. The calcium permeability changes may be brought about by

other vitamin D-induced proteins (26), but such proteins would have to be unaffected by cycloheximide and actinomycin D.

Of critical importance to the eventual definition of the mechanism of $1,25(OH)_2D_3$-stimulated calcium transport will be the morphologic and/or compositional changes in membrane associated with altered calcium permeability. More precise intracellular localization of CaBP will be very helpful to discern its biological function.

Summary. $1,25(OH)_2D_3$ increases cell permeability to calcium. This increase is not mediated by proteins sensitive to cycloheximide or actinomycin D inhibition. We propose that CaBP may associate with intracellular membranes and organelles to prevent intracellular calcium accumulation and the potential cytotoxic effects of such accumulation. In support of this hypothesis, the amount of mitochondrial mineralization in chick intestinal cells was markedly increased by $1,25(OH)_2D_3$ treatment when CaBP synthesis was simultaneously blocked by cycloheximide treatment. Mineral in membrane vesicles was increased by $1,25(OH)_2D_3$ treatment, but was blocked by simultaneous treatment with cycloheximide.

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Footnote

[1] Present address: Pathology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD 21701.

[2] Present address: Clinical Research Service, Fitzsimons Army Medical Center, Denver, Colorado 80240.

Figure Legends

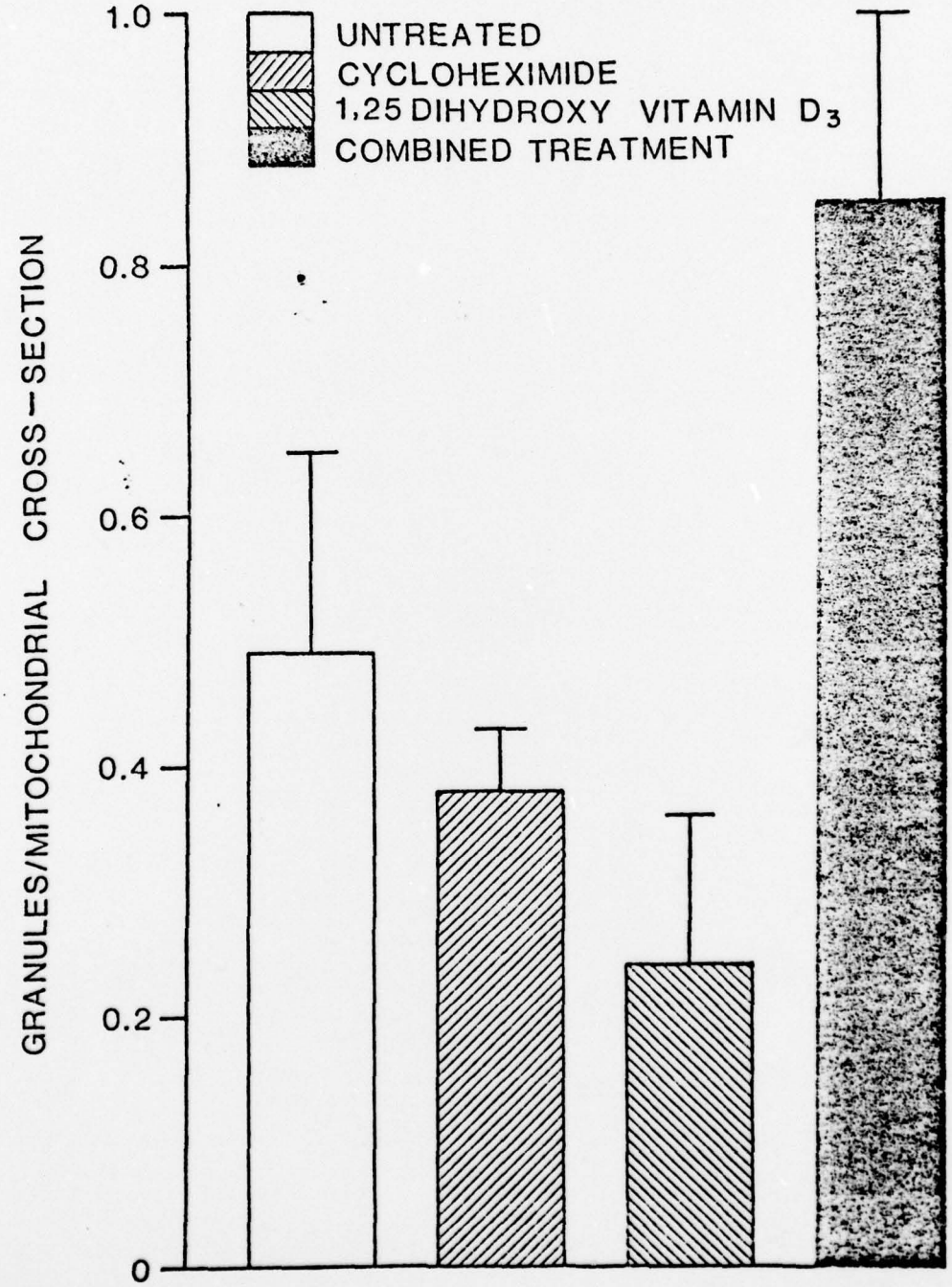
FIG. 1. Mitochondrial mineralization in the epithelial cells of intestinal villi 18 hr after treatment with carrier vehicles only, cycloheximide only, $1,25(OH)_2D_3$ only or both cycloheximide and $1,25(OH)_2D_3$. *Means ± SE of the mean for the 3 chicks are shown.*

FIG. 2. Epithelial cells of intestinal villi 18 hr after treatment with carrier vehicles only (A), cycloheximide only (B), $1,25(OH)_2D_3$ only (C) or combined cycloheximide and $1,25(OH)_2D_3$ (D). Mitochondria (mito), membrane vesicles (ves) and mineral granules (g) are indicated.

FIG. 3. Mineral content of membrane vesicles in the epithelial cells of intestinal villi 18 hr after treatment with carrier vehicles only, cycloheximide only, $1,25(OH)_2D_3$ only or combined cycloheximide and $1,25(OH)_2D_3$. Means ± SE of the mean for the 3 chicks are shown.

FIG. 4. Proposed model to represent the actions of $1,25(OH)_2D_3$ on the intestinal epithelial cell. Three distinct actions of $1,25(OH)_2D_3$ proposed are: (1) enhances synthesis of alkaline phosphatase (Alk. Pase) from preexistent mRNA, (2) induction of mRNA for CaBP, and (3) a direct or cytosol-mediated effect on membrane permeability to calcium that is independent of new protein synthesis. We propose that CaBP either prevents calcium from associating with mitochondria or enhances calcium dissociation from mitochondria (M.C.) and enhances calcium uptake by membrane vesicles. The circles inside the mitochondria represent mineralization granules.

Fig. 1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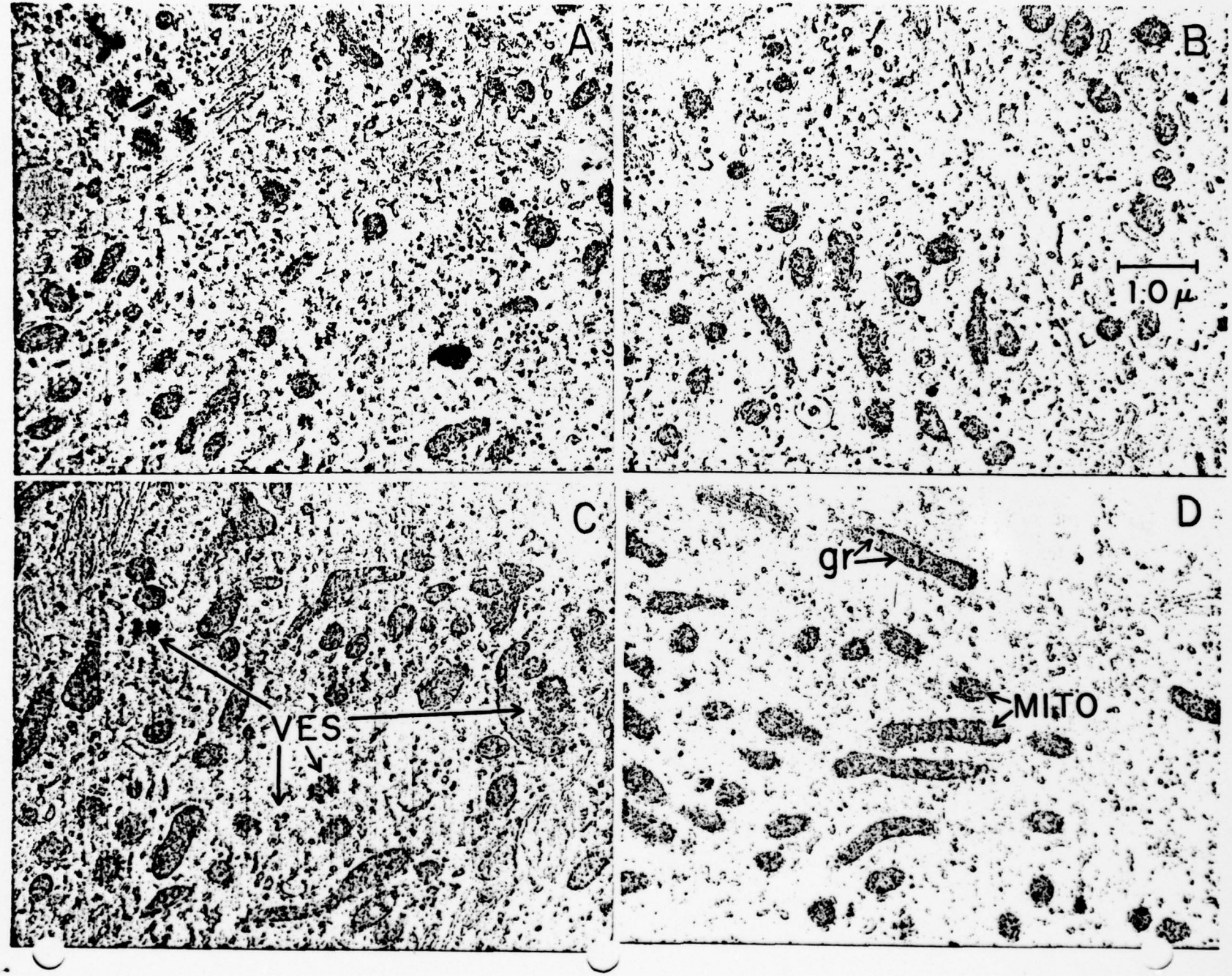
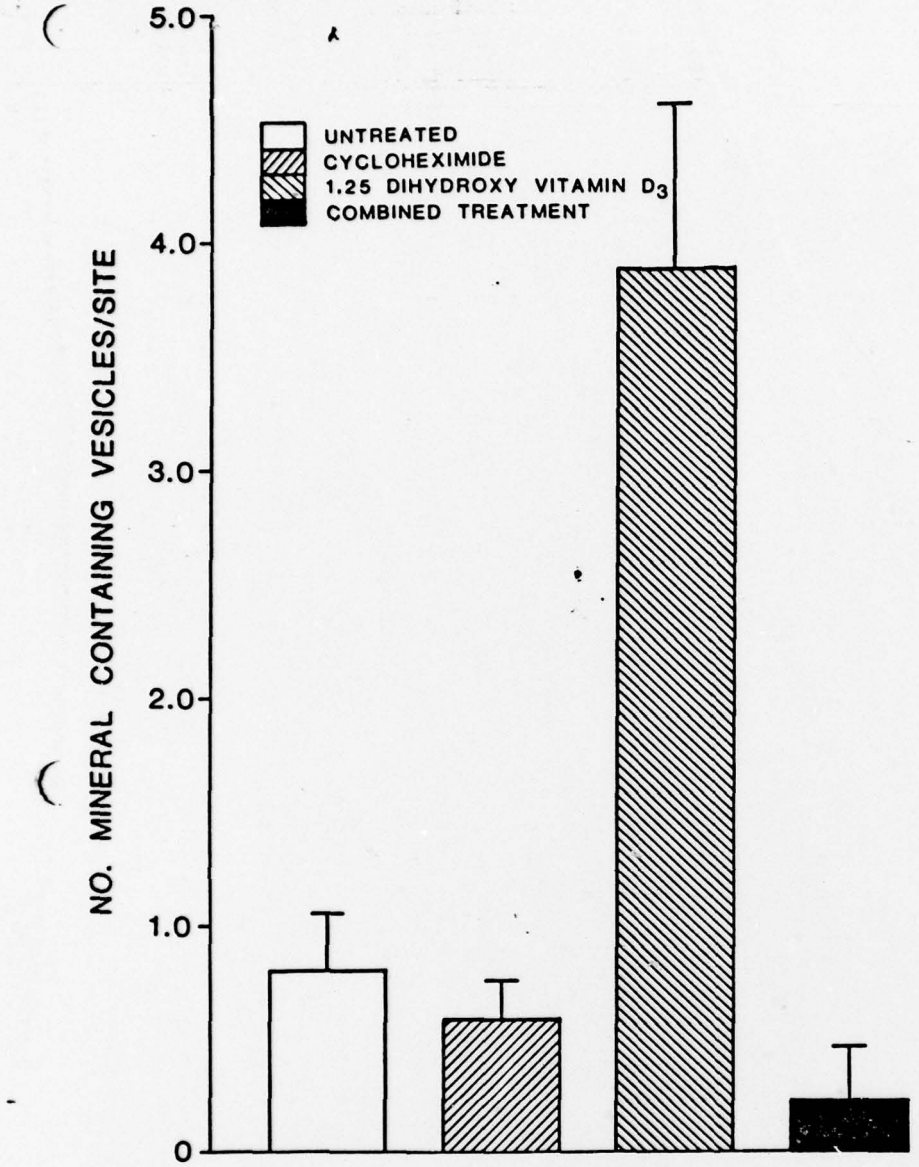


Fig. 2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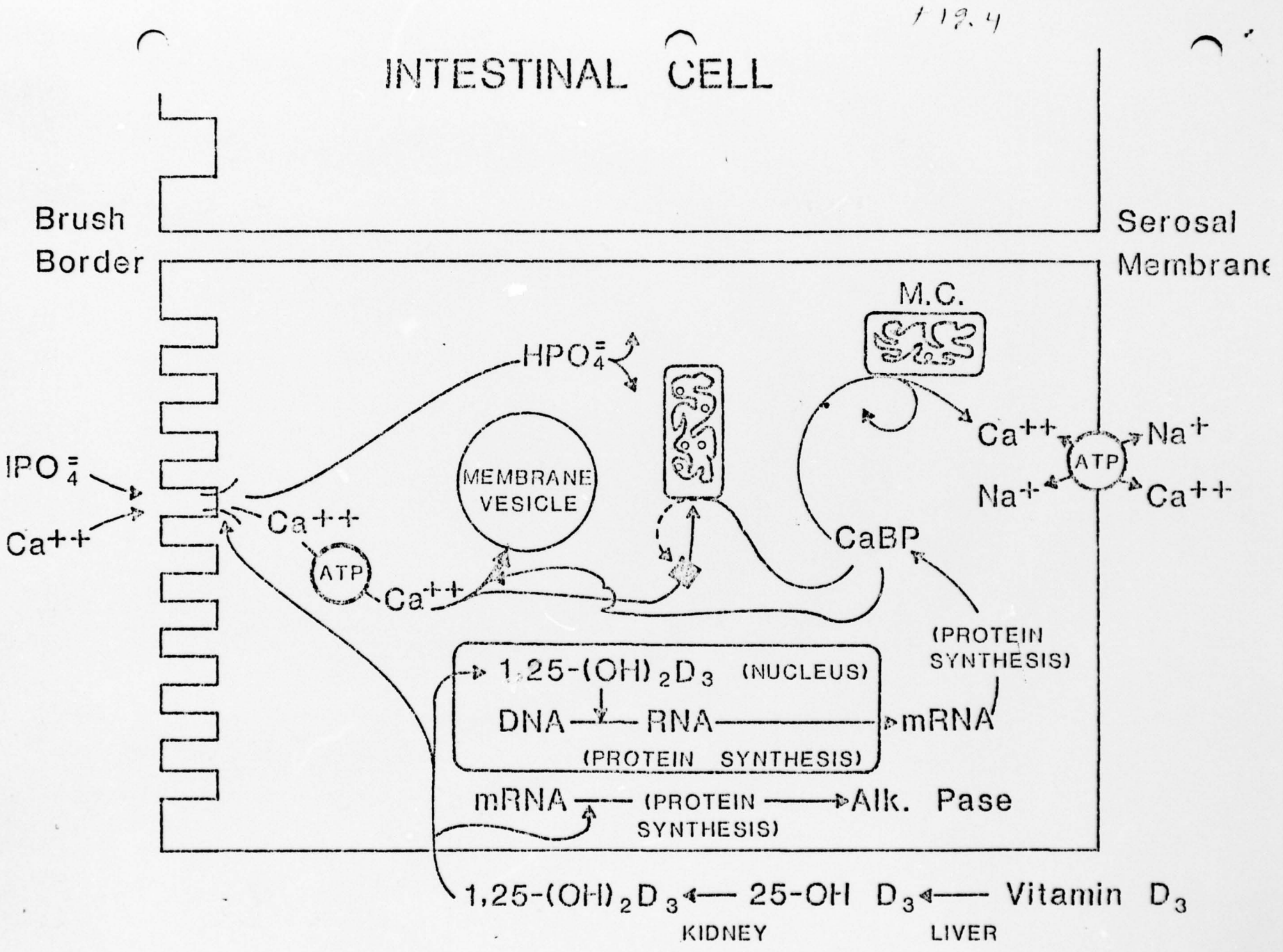
COMBINED TREATMENT

Fig. 3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Brush Border
Serosal Membrane
M.C.
HPO4=
IPO4=
Ca++
Ca++
ATP
Ca++
MEMBRANE VESICLE
Ca++
ATP
Na+
Na+
Ca++
CaBP
(PROTEIN SYNTHESIS)
1,25-(OH)2D3 (NUCLEUS)
DNA
RNA
mRNA
(PROTEIN SYNTHESIS)
mRNA
(PROTEIN SYNTHESIS)
Alk. Pase
1,25-(OH)2D3
25-OH D3
Vitamin D3
KIDNEY
LIVER

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PAUL W. MELLICK, AND DANIEL D. BIKLE

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The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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